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Section 8d

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"Characterization of 14C Complexes and Metabolites of TDI in the Blood of Exposed Rats."

Name of Chemical Substance:	14C-Toluene-2,4-Diisocyanate
Chemical Abstract Service Number:	584-84-9
Description of Study:	Rat inhalation exposure 14C-Toluene-2,4-Diisocyanate to determine "in vivo" fate.
Date of Issue:	July 5, 1994
Name & Address of Testing Organization:	The Laboratory of Dr. W.E. Brown Dept. of Biological Sciences Carnegie Mellon University 440 Fifth Avenue Pittsburgh, Pennsylvania 15213

Very truly yours,

R. K. Rigger
Managing Director

RKR/sha
Enclosure

Study Title

Characterization of ^{14}C Complexes and Metabolites of TDI in the Blood of Exposed Rats

Author(s)

A.L. Kennedy and W.E. Brown

Study Completion Date

September, 1993

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Compound: **^{14}C-Toluene-2,4-Diisocyanate**

Title: **Characterization of ^{14}C Complexes and Metabolites of TDI in the Blood of Exposed Rats**

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SUMMARY

Inhalation exposure to toluene diisocyanate (TDI) can result in a variety of airway diseases. Concern has been expressed that a putative carcinogenic potential of TDI exists as a result of the formation of toluenediamine (TDA) by hydrolysis of the isocyanate in the body. Results from long-term bioassays (TDI rat and mice inhalation versus TDI rat gavage) are contradictory and discrepancies do exist concerning the interpretation of adverse effects. This study was performed to analyze the distribution and distribution of radioactively-labeled TDI using vapor exposure in a rat model system. Rats were exposed to ^{14}C-TDI vapors at concentrations ranging from 0.026-0.821 ppm. All tissues examined showed detectable quantities of radioactivity, with the airways gastrointestinal system and blood having the highest levels (μgEq/g). The concentration of radioactivity in the bloodstream after 4 hours of exposure increased linearly with respect to dose. The majority (74-79%) of the label associated with the blood was recovered in the plasma, and of this, 97-100% of the ^{14}C existed in the form of biomolecular conjugates. Analysis of stomach contents shows that the majority of the label is also associated with high (> 10 kDa) molecular weight species. While a larger percentage (28%) of the label in the stomach contents is found in the low molecular weight fraction relative to blood, this low molecular weight labelled material represents at least eight different components. Thus, over the vapor exposure concentrations and time tested, it appears that conjugation is the predominant reaction and that free TDA is not a primary *in vivo* reaction product.

INTRODUCTION

Isocyanates are a group of highly reactive compounds which are important in a number of industrial applications. The forms predominantly used include: toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI) and hexamethylene diisocyanate (HDI). A complex mixture of respiratory diseases has been associated with diisocyanate exposure (Patterson *et al.*, 1987) and these diseases are not completely understood at the molecular level (Kennedy and Brown, 1992). In addition to the effects of diisocyanates on the respiratory system, there has been an increased interest in investigating the carcinogenic potential of these compounds based on possible hydrolysis and production of diamines. Of particular concern is the *in vivo* fate of TDI. This has been fueled by the findings that toluenediamine (TDA), a hydrolysis product of TDI, is carcinogenic in rodents and tests positively in *in vitro* models (NCI, 1979). TDI, itself, has been tested using inhalation (Loeser, 1983) and gavage administration in corn oil (Dieter *et al.*, 1990); however, a consensus regarding its carcinogenic potential has not been reached. While chronic inhalation, the exposure relevant at the workplace, did not result in tumor formation in rats and mice, an increased rate of certain tumors in rats was found after gavage administration of TDI in corn oil. When TDI is introduced into an aqueous environment, the potential for diamine production exists. After complete hydrolysis of biological samples, Skarping and co-workers (1991) have shown that TDA can be detected and quantitated in the hydrolyzed plasma and urine samples from TDI-exposed workers. Similarly, Rosenberg and Savolainen (1985) have demonstrated the presence of TDA in hydrolyzed urine samples from rats following TDI exposure. The fact that these analytical methods are based on the prior hydrolysis of the biological samples and that the isocyanate can react directly with biological macromolecules resulting in hydrolysable adducts, it is not possible to determine the original state of the tolyl group in the organism. Through hydrolysis experiments alone, it is therefore, difficult to assess the risk associated with TDA production as a result of TDI exposure. Thus, it is important

to directly determine the biochemical reactivity and fate of these compounds, particularly following inhalation exposure.

A number of studies have been conducted to investigate the uptake and metabolic fate of TDI in animals using radioisotopic techniques (Saclay, 1977; Stoltz *et al.*, 1987; Kennedy, *et al.*, 1989; Dieter *et al.*, 1990, and Timchalk, *et al.*, 1993). These studies have reported the general distribution of radioactivity following exposure and subsequent clearance. All have consistently shown that the radioactivity reaches the bloodstream in some form. Previous work in this laboratory has demonstrated the rapid uptake of ^{14}C into the bloodstream of guinea pigs exposed to TDI at concentrations ranging from 0.004 ppm to 0.15 ppm for periods of 1-5 hr (Kennedy, *et al.*, 1989). Following inhalation exposure at these levels, which are in the range of the current workplace limit values, a linear correlation is seen between exposure and blood level of radioactivity. ^{14}C was detected in all tissues and body fluids examined following these exposures. Additional research in this laboratory has involved the use of radioactive isocyanate exposure of guinea pigs to characterize the reaction products which are found in the respiratory tract and blood (Hill, 1986; Kennedy, 1990). In an initial biochemical study done by Hill (1986) it was shown that under the exposure condition tested, TDI underwent selective protein modification reactions *in vivo* in a guinea pig model. This selective reactivity was confirmed over a range of relatively low TDI vapor concentrations also tested in the guinea pig model (Kennedy, 1990). Metabolism studies in rats following TDI inhalation have also supported the *in vivo* reactivity of TDI with biomolecules based on the fact that only a small percentage of the dose can be recovered as either TDA itself or TDA derivatives (Timchalk, *et al.*, 1993). The study reported here was performed to further characterize the biochemical events following inhalation exposure to TDI in a rat model to aid the interpretation of carcinogenic risk.

EXPERIMENTAL

General Design and Rationale

For these experiments, the ^{14}C-TDI was administered via heads-only inhalation for 4 hours. Groups of four male rats were used per exposure concentration. Three exposure concentrations were tested to address the dose relationship of the biochemical state of the ^{14}C in the bloodstream. The target concentrations of 0.02 and 0.2 ppm were chosen to include values close to the levels tested in in the chronic bioassay. The high concentration (0.8-1.0 ppm) was chosen since it was reasonable to generate and it enhanced the analytical sensitivity without causing an acute response. All animals were euthanized immediately following exposure and the tissues and fluids were collected for analysis. Characterization of distribution and reaction were performed as outlined in Figure 1.

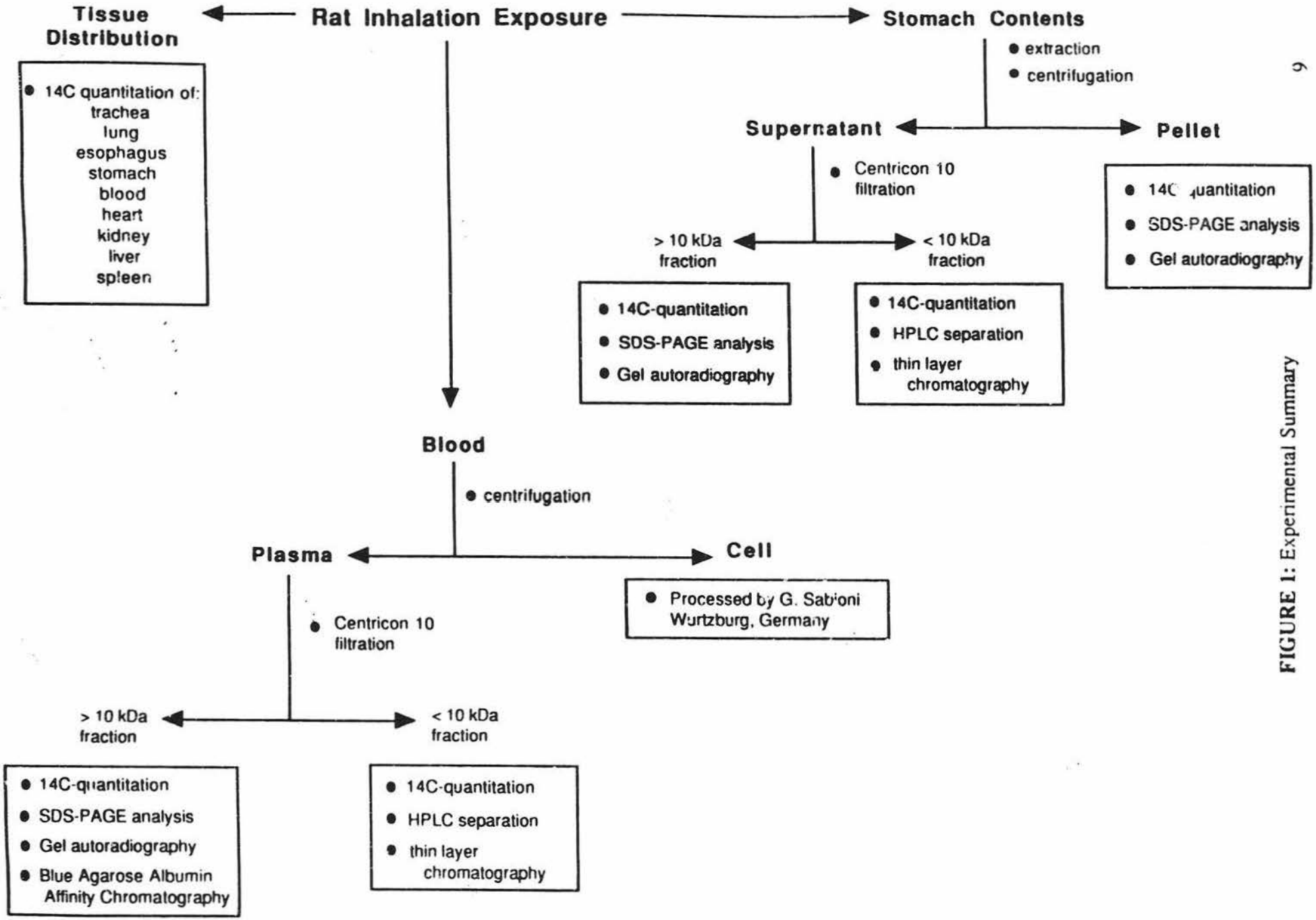


FIGURE 1: Experimental Summary

MATERIALS AND METHODS

Chemicals. 2,4-[^{14}C]TDI (12 mCi/mmole) was synthesized by New England Nuclear (NEN; Boston, MA) with the ^{14}C incorporated in the benzene ring. Gas chromatographic analysis provided by NEN showed the radiochemical to be 99% pure. Derivatization and HPLC analysis of the sample confirmed the reactivity and purity as 2,4 TDI (See Quantitation of Isocyanate Concentrations section). Unlabeled 2,4 TDI was purchased from Fluka Chemical Co. and was used as a reference compound as well as to establish the instrument settings for the target exposure concentrations.

Animals. Male, Fischer 344 rats (150 - 200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were held in an animal room for at least seven days prior to exposure with food and water provided *ad libitum*. Sixteen rats were used in this study. Three exposure levels were tested with four animals at each concentration. Four additional animals served as controls.

Head-Only Rat Exposure System. A continuous airflow system was used for all radioactive TDI exposures. A four port, glass chamber was constructed with four detachable glass tubes to hold the animals for heads-only exposure. Dental dam collars were fitted around the rats' heads to minimize body contamination and possible dermal absorption. The radioactive TDI was shipped in sealed, glass mini-vials previously described (Ferguson *et al.*, 1988). To generate the TDI vapor, house air was dried, filtered and delivered over the liquid in the vial once the internal glass septum was broken with a needle. Air flow was controlled by an appropriate flowmeter. Choice of flowmeters and rate settings, ranging from 120 ml/min to 2 liters/min, for the radioactive experiments was determined using identical, mini-vials containing unlabeled TDI. During vapor generation, the vial was submerged in a constant temperature, paraffin oil bath

which was maintained at 55°C. A 20 gauge needle also penetrated the top of the vial to deliver the vapor to the exposure chamber. The vapor was drawn into the system by a vacuum pump equipped with a valve and flowmeter to regulate the exhaust airflow from the exposure chamber. Exhaust rates varied between 10 and 20 liters/min depending on the isocyanate concentration desired.

Quantitation of Isocyanate Concentrations. The quantitation of isocyanate concentrations in the system was performed throughout the 4 hours of exposure by the periodic sampling of the chamber atmosphere using two different assays, as well as the scintillation analysis of assay fluid. One assay used for the determination of isocyanate concentration was the Marcali method (Marcali, 1957) as modified by NIOSH (1978). The Marcali determination involves the hydrolysis of isocyanate to toluenediamine and conversion to a colorimetric product. Air samples were bubbled into an impinger containing the acidic Marcali trapping solution for periods of 5-15 min depending on the target exposure concentration. Samples were processed and then quantitated by absorbance at 550 nm relative to a 2,4 TDA calibration curve. An aliquot of this solution was also counted in ACS liquid scintillant (Amersham) for calculation of isocyanate content based on the specific activity of the original compound. The third quantitation method involved the derivatization of the reactive isocyanate with p-nitrobenzoylpropylamine (PNBPA; Regis Chemical Co.) (Schroeder and Moore, 1985) which was immobilized on glass fiber filters. Air samples were drawn through the coated glass fiber filter cassettes at a rate of 2 liters per minute for periods of 5-15 min. The filters were extracted in acetonitrile and an aliquot of the filter extract was analyzed by reversed phase HPLC and the area of the derivative peak was quantitated relative to a calibration curve.

Collection of Terminal Blood, Body Fluids and Tissues. Animals were euthanized (2 ml Beuthanasia 50 mg/ml, ip) immediately upon termination of the exposure. Terminal blood samples were collected via cardiac puncture and immediately mixed in a Vacutainer tube (Becton Dickinson) containing sodium citrate as an anticoagulant. Two, 200 µl aliquots were placed in glass vials to determine ^{14}C content. To each vial, 2.4 ml of NCS tissue solubilizer (Amersham) was added and the suspension was heated at 50°C for 20 min. An aliquot of 0.8 ml of a 20% benzoyl peroxide solution was added followed by an incubation at 50°C for an additional 30 min to decolorize the samples. After cooling to room temperature, organic scintillant (toluene, 2,5-diphenyloxazole, and 1,4,-bis 2-(5-phenyloxazolyl)benzene) was added to bring the final volume to 20 ml. To reduce the level of background radioactivity due to chemiluminescence, the samples were stored in the dark for at least 24 hr before scintillation analysis. Total radioactivity was calculated on both a cpm/ml and microgram equivalents of tolyl group per milliliter (µgEq/ml) of blood basis.

Trachea, lung, esophagus, stomach and contents, kidney, heart, spleen and liver were dissected from each of the exposed animals as well as four control animals. The stomach contents were removed from the organ and stored separately. All materials were immediately frozen at -60°C until further analyzed.

Tissue Solubilization and Quantitation of Associated Radioactivity. Tissue solubilization and quantitation of ^{14}C content were performed on representative tissue fragments from the major organs of all exposed and control animals. The fragments were weighed and transferred to glass scintillation vials. Hyamine hydroxide (ICN) was added to a volume six times the total sample weight or a minimal amount of 1 ml and was incubated at 50°C with agitation for 24 hr. Samples were cooled and acidified to pH 6-7 with glacial acetic acid. Scintillant was added and the samples were counted. Results

were corrected for background and estimated blood content and the values were normalized on a µgEq per gram tissue basis.

Plasma and Cell Isolation. Plasma and cellular blood components were separated by centrifugation of terminal blood samples at 478 x g for 5 min. Plasma was removed and stored at -60°C. The cellular fractions were stored at 4°C to avoid freeze-fracturing of the cell membranes. To determine the relative distribution of radioactivity in cellular and plasma fractions, 100 µl aliquots of plasma were counted directly in 5 ml Cytoscint ES (ICN), whereas the blood cells were washed with phosphate buffered saline (PBS) to remove extraneous plasma and then solubilized, as described for whole blood, before counting. Distribution of radioactivity in each fraction was calculated as a percentage of the total blood-associated radioactivity.

Molecular Size Fractionation of Plasma. The relative distribution of high and low molecular weight components in plasma was determined by scintillation analysis following separation by molecular size fractionation. This was performed on 0.2 ml aliquots of plasma diluted to 1 ml with PBS, pH 7.4, using Centricon 10 microconcentrators (Amicon). Samples were spun for 40 min at 5000 x g. The retentate was washed with an additional 1 ml of buffer and recentrifuged. Retentates were recovered by centrifugation at 746 x g for 4 min. Filtrates and retentates were assayed for distribution of radioactivity.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis. Plasma retentate samples, as well as stomach content fractions, were subjected to gel electrophoresis following the procedure of Laemmli (Laemmli, 1970) using a 10% acrylamide resolving gel. The gels were stained with Coomassie blue and destained in an acetic acid/ isopropanol/ water solution.

Gel Autoradiography. Following the destaining procedure, each gel was dried using the BioWrap system (BioDesign, Inc.). The dried gels were then exposed to X-Omat AR film (Kodak) at -60°C. The film was developed following the manufacturer's specifications.

Blue Agarose Affinity Chromatography. Plasma retentate samples were loaded onto a Reactive blue 4-cross-linked agarose affinity column (Sigma Chemical Co.) equillibrated with 0.01M Tris HCl, pH 7.8 buffer. Fractions (5 ml) were collected and the absorbance at 280 nm as well as fraction conductivity were assayed. The bound protein was eluted by a step gradient to 0.05M Tris HCl, pH 7.5 buffer containing 0.2M NaSCN. Aliquots (100 µl) of each fraction were dissolved in 5 ml Cytoscint ES (ICN) and counted to quantitate ^{14}C content.

Thin Layer Chromatography of Plasma Filtrate Fractions. Plasma filtrate (<10kDa) fractions were analyzed by thin layer chromatography (TLC) using SilG plates (Brinkmann) and an acidic solvent system composed of 40 ml acetic acid, 10 ml butanol and 10 ml deionized water. 20 µl of each sample was spotted onto the plate and developed in the solvent chamber. 2 µg of toluenediamine (Aldrich) was included as a reference compound. Rf values were determined for all samples.

Stomach Content Extraction and Analysis. The stomach contents were mixed and a sample from each animal was weighed and transferred to a microfuge tube. The solid was resuspended in 1 ml PBS and incubated 5 minutes with shaking. Supernatants were recovered by centrifugation for 10 min at 12,000 x g. Aliquots of the supernatants were dissolved in Cytoscint ES and counted to determine ^{14}C content. The pellets were washed six times by the same procedure. Pooled supernatants were fractionated into

retentate (>10 kDa) and filtrate (<10kDa) fractions as described above for the plasma samples. High molecular weight fraction components and the distribution of radioactive materials were determined through SDS-PAGE and autoradiography. Filtrate samples were analyzed through reverse phase high pressure liquid chromatography (RP-HPLC) on a Econosil C_{18} column (Alltech) with a 10 micron pore size. Products were eluted with a linear gradient from 0 to 80% methanol with 2% PicB7 low UV reagent (Waters). Absorbance was monitored at 214 nm. Fractions were collected at 30 second intervals and were counted to determine radioactive content.

RESULTS

Determination of Radiochemical Purity and Specific Activity.

Gas chromatographic analysis was performed by NEN on the ^{14}C TDI which demonstrated a compound purity of 99% and a specific activity of 12 mCi/mmole (Figure 2). Reversed phase HPLC analysis was also conducted on the radiochemical following derivatization with PNBPA (Figure 3A). Fractions were collected across the profile and counted (Figure 3B). For each exposure, 95-99% of the injected radioactivity was recovered with 98-99% of the recovered cpms in the 2,4 TDI derivative peak (Fractions 3+4) (Table 1). Retention times of unlabeled 2,4-TDI as well as 2,6-TDI, Mondur TD80 (80% 2,4 isomer / 20 % 2,6 isomer) (Miles) and toluenediamine (TDA) were analyzed under identical conditions (Kennedy, *et al.*, 1989). This analysis showed that the radiolabeled peak co-migrated with 2,4-TDI and did not contain other contaminating compounds such as the 2,6 isomer or the hydrolysis product, TDA. A concentration calibration curve was generated with unlabeled 2,4-TDI (Fluka) and the peak areas were used to determine the concentration of TDI in the radioactive samples. These analyses confirmed the exposure compound purity and concentration. The ability of the radioactive compound to react with the PNBPA demonstrated that the chemical form used for animal exposure was reactive isocyanate.

FIGURE 2: (Following Page) Photocopy of GC analysis of synthetic 14C-TDI starting material provided by New England Nuclear. (Data provided by NEN).

GAS CHROMATOGRAPHY

Compound 2,4-Toluenediisocyanate

Catalog No. NEC-999 Lot No. 2732-123

Column: 2.5% OV-225 6'x1/8"

TEMP: COLUMN 100(2)-16-200(16) °C

TEMP: INJ. 250 °C DET. 250 °C TRANSFER 250 °C

CARRIER GAS ARGON Flow Rate 30/20/60 ml/min. col/mu/total

DETECTOR F.I.D. H_2 35 ml/min. AIR 350 ml/min.

SENSITIVITY 1(1) RECORDER SPEED 5.0 mm/min.

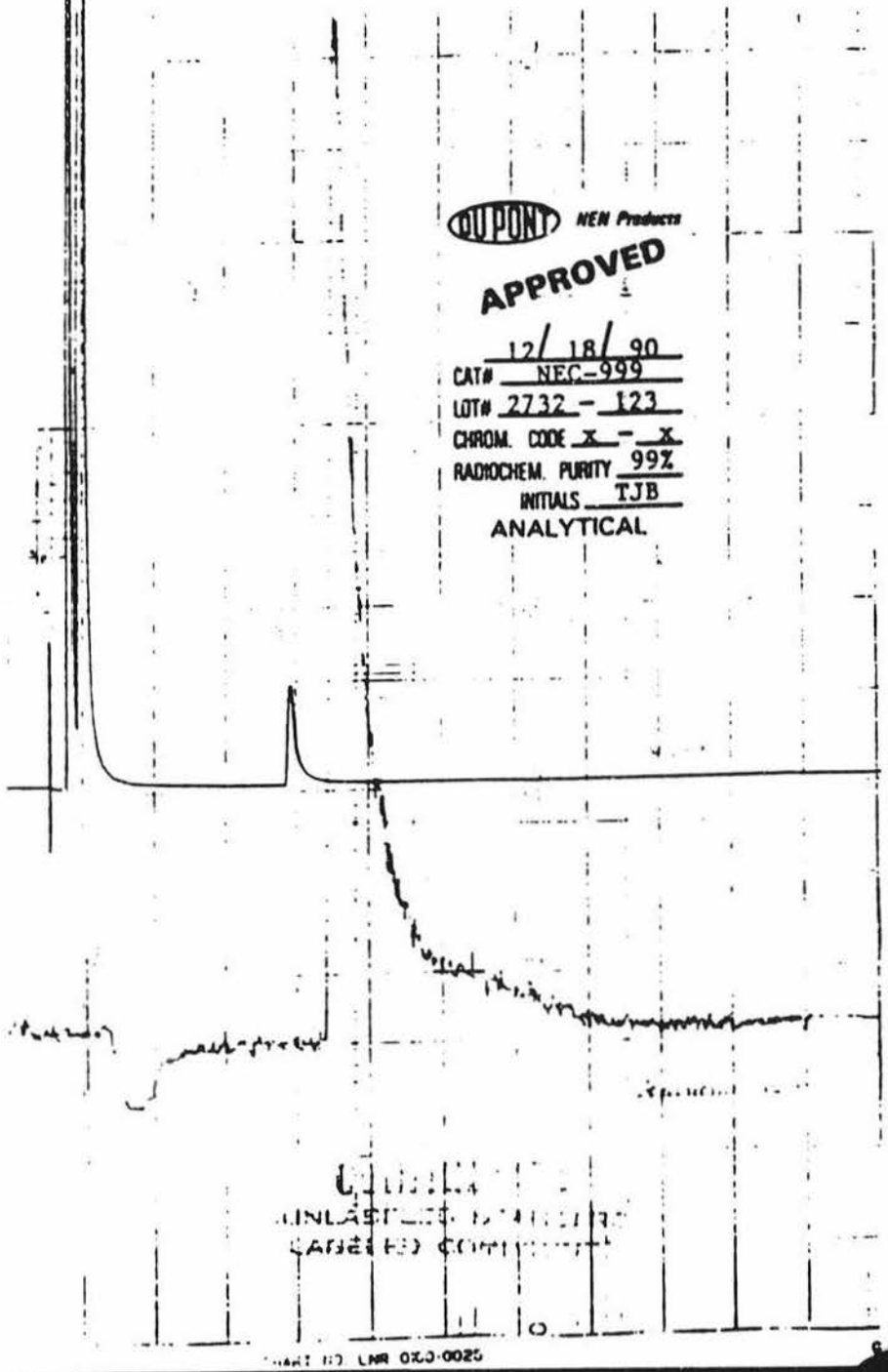
SOLVENT CH_2Cl_2/Tol (1/1) SAMPLE SIZE .2uL

Ratemeter Full Scale 100,000 cpm. COLUMN NO.

Instrument No. 1 Operator TJB Date 12/18/90

COMMENTS:

ACTIVITY PEAK DISPLACED BY APPROXIMATELY 30 SECONDS FROM MASS PEAK DUE TO ADDITIONAL PATH LENGTH THROUGH COMBUSTION TUBE AND PROPORTIONAL FLOW COUNTER



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LOT# 2732 - 123

CHROM. CODE X - X

RADIOCHEM. PURITY 99%

INITIALS TJB

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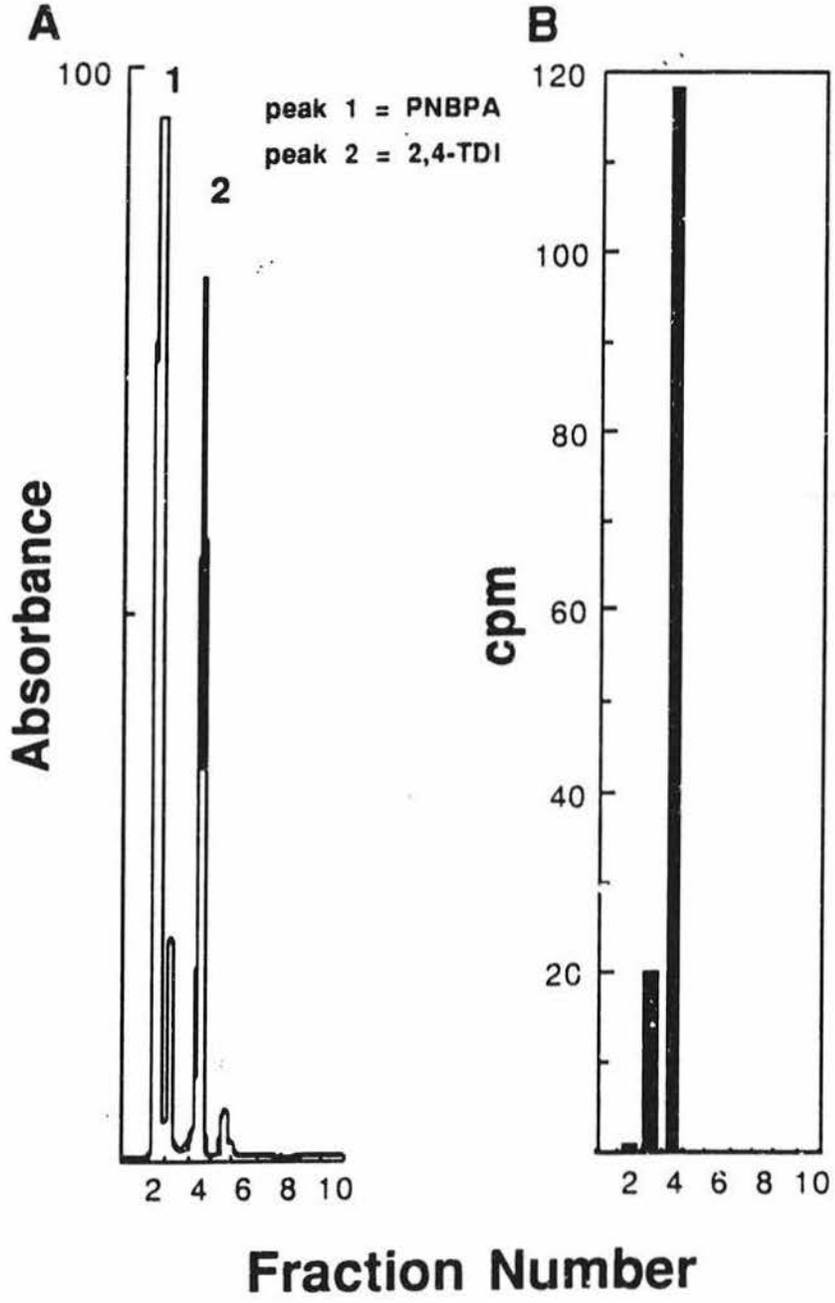


FIGURE 3: RP-HPLC analysis of PNBPA derivatized 14C-TDI. (A) Absorbance profile at 254 nm of column effluent. (B) cpm of each one minute fraction collected during elution of PNBPA-derivatized 14C-TDI.

TABLE 1. Characterization of Exposure Compound: Distribution of Radioactive Components in PNBPA Filter Extracts.

Exposure #	n	% of total cpm recovered (avg ± S.D.)	% of cpm recovered in Fx2 (avg ± S.D.)	% of cpm recovered in Fx(3+4) (avg ± S.D.)
1	3	98.2 ± 5.3	0.46 ± 0.2	98.7 ± 1.0
2	3	95.1 ± 1.0	0.68 ± 0.1	99.0 ± 0.3
3	4	98.6 ± 4.0	0.38 ± 0.1	99.3 ± 0.2

Quantitation of Isocyanate Exposure Concentrations.

A series of ^{14}C TDI exposures was conducted over a range of concentrations as given in Table 2. Three methods were used to monitor the exposure atmospheres: the Marcali assay, scintillation analysis of Marcali trapping solutions, and HPLC analysis of PNBPA-derivatized isocyanate. Table 2 summarizes the compiled data available for each experiment. Multiple samples, were collected during the 4 hr exposures at approximately 30 min intervals. Figure 4 is included to demonstrate the consistency of the atmospheric concentrations over the exposure periods. The greatest degree of fluctuation was observed at the highest concentration. The concentration determinations from all methods were averaged. This yielded average exposure concentrations of 0.026, 0.143 and 0.821 ppm for the experimental series. Table 3 summarizes the exposure data.

TABLE 2: Quantitation of Isocyanate Atmospheric Concentration

Exposure #	Marcali (A550)			Marcali (cpm)			PNBPA (HPLC)			Average Conc. (ppm)	SD
	n[a]	ppm average	SD[b]	n	ppm average	SD	n	ppm average	SD		
1	9	0.016	0.004	9	0.027	0.003	10	0.037	0.006	0.026	0.010
2	9	0.088	0.022	9	0.119	0.027	9	0.223	0.019	0.143	0.071
3	9	0.77	0.099	9	0.90	0.125	9	0.790	0.130	0.821	0.070

[a] n = number of samples taken during each exposure
[b]SD = standard deviation

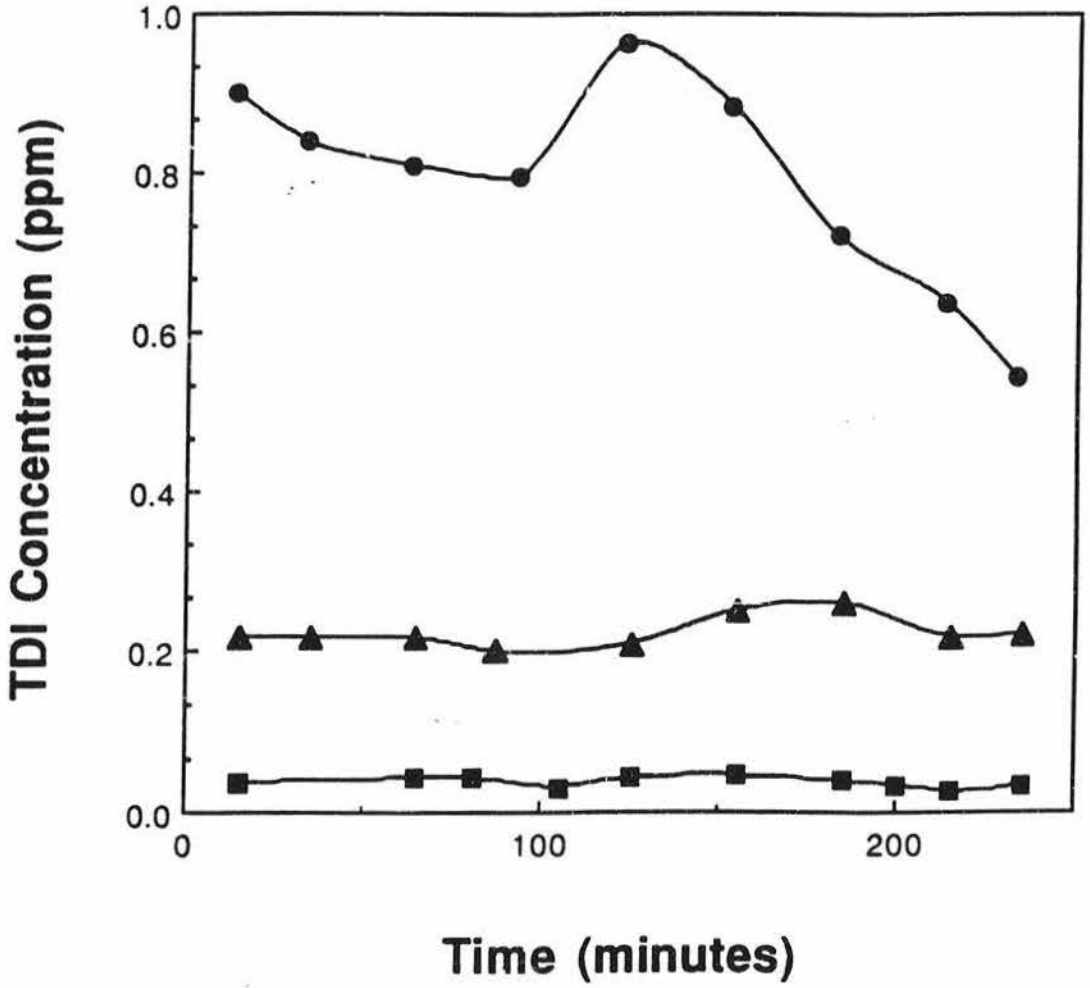


FIGURE 4: Variation of TDI concentration during exposure. Air samples were trapped on PNBPA impregnated filters at the indicated times during each exposure and the samples were analyzed for TDI concentration as described in the Methods Section.

TABLE 3: ^{14}C-TDI Exposure Summary

Exp. #	Animal #	Animal Weight (g)	Target TDI Conc. (ppm)	Actual TDI Conc. (ppm)	Duration of Exposure (hrs)	Time of euthanasia post-exposure
1	1 2 3 4	183 173 172 189	0.02	0.026 ± 0.01	4	< 1 hr
2	5 6 7 8	172 178 172 180	0.20	0.143 ± 0.07	4	< 1 hr
3	9 10 11 12	172 181 184 187	0.8-1.0	0.82 ± 0.07	4	< 1 hr
C	C17 C18 C19 C20	208 206 197 199	-	-	-	

Based on the exposure concentrations, an estimated dose for each exposure group was calculated, assuming 100% retention of the reactive vapor using the following equation: concentration (mg/ml) * time * tidal volume * frequency of respiration. A summary of the results of these calculations is given in Table 4.

TABLE 4: Calculation of Estimated, Inhaled Dose

ppm	Concentration (mg/ml)	Time (min)	VT (ml)	f (breaths/min)	dose[a] (mg)	dose[b] (cpm)
0.026	1.9×10^{-7}	240	1.5	100	6.7×10^{-3}	8.5×10^{5}
0.143	1.0×10^{-6}	240	1.5	100	3.7×10^{-2}	4.7×10^{6}
0.821	5.9×10^{-6}	240	1.5	100	2.1×10^{-1}	2.7×10^{7}

[a] $Dose_{(mg)} = C_{(mg/ml)} * T_{(min)} * VT_{(ml)} * f_{(breaths/min)}$

[b] $Dose_{(cpm)} = Dose_{(mg)} *$ Mol Wt $*$ Specific Activity $*$ dpm/mCi $*$ Counting Efficiency

Distribution of ^{14}C in Tissues of TDI-Exposed Rats.

Immediately following each exposure, the experimental group of four rats was euthanized and major organs were collected for scintillation analysis. The results of the digestion of tissue fragments from the trachea, lung, esophagus, stomach contents, heart, liver, kidney and spleen, as well as scintillation analysis of blood, for all three exposure concentrations are represented in Figure 5. The inset includes non-airway tissues with an expanded ordinate axis. Variability within the exposure groups is shown (error bars). As can be expected, the highest levels of radioactivity (µgEq/g) were associated with the airway tissues. In addition, some form of the radioactivity was found associated with the other organs analyzed. The specific activity (µgEq/g) of the ^{14}C in all tissues increased with exposure concentration. The percentage of the calculated, estimated dose for each tissue is given in Table 5. The tissues are presented in three groupings: airway materials, gastrointestinal materials and blood and other systemic organs; each group showing decreasing levels of labeling as the material went through the system.

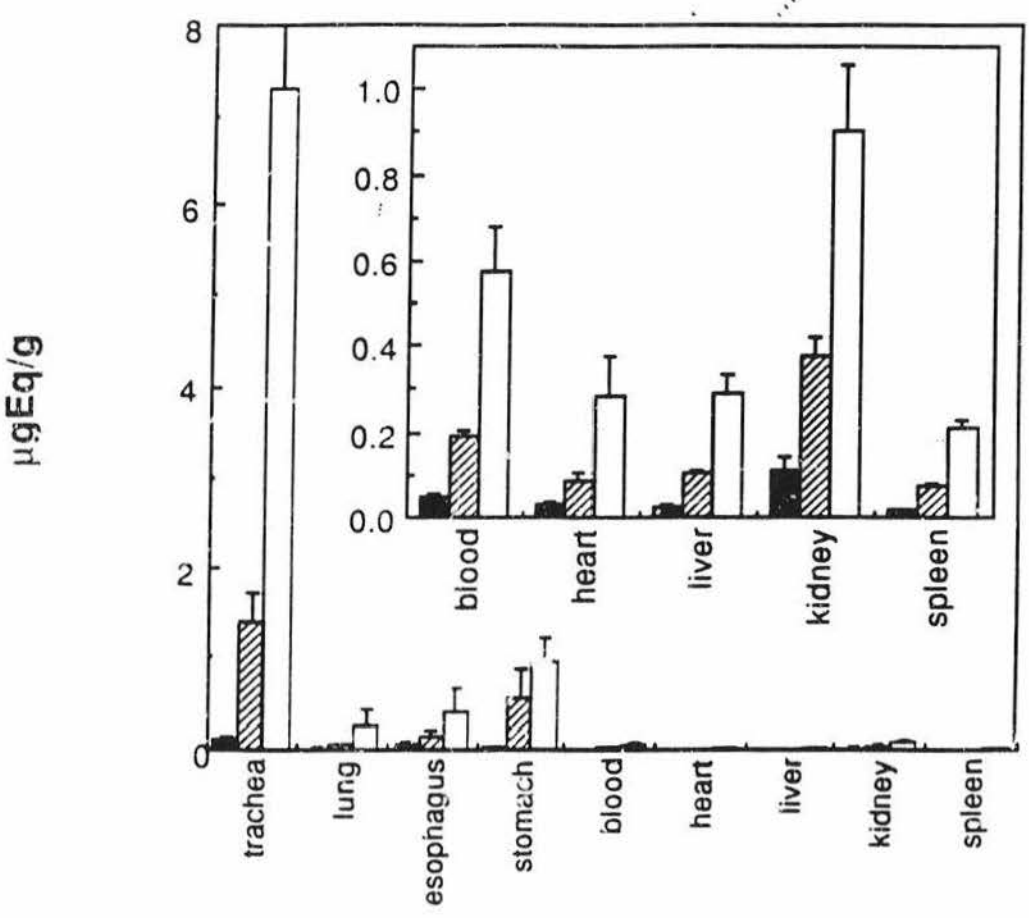


FIGURE 5: Distribution of Radioactivity in Tissues of Rats Following Inhalation Exposure to ^{14}C-TDI. Tissue distribution of tolyl group expressed as µgEq/g tissue immediately following ^{14}C-TDI inhalation exposure of rats as a function of exposure concentration (0.026 ppm - filled bars; 0.143 ppm - hatched bars; 0.821 ppm - open bars). Error bars indicate standard deviation of data from the exposure group of 4 animals. Inset is included as ordinant expansion for clearer representation of selected data.

TABLE 5: Tissue Distribution of Radioactivity Following ^{14}C-TDI Exposure.

Organ System	Tissue	0.026 ppm		0.143 ppm		0.821 ppm	
		µgEq/gm average ± SD	% 0f Dose ± SD	µgEq/gm average ± SD	% of Dose ± SD	µgEq/gm average ± SD	% of Dose ± SD
Airway	Trachea	1.045 ± 0.346	0.39 ± 0.16	14.25 ± 3.21	0.79 ± 0.18	73.02 ± 40.08	0.63 ± 0.49
	Lung	0.124 ± 0.031	1.42 ± 0.36	0.431 ± 0.108	0.89 ± 0.22	2.687 ± 1.640	0.88 ± 0.49
Gastro-Intestinal	Esophagus	0.453 ± 0.273	0.70 ± 0.44	1.437 ± 0.308	0.39 ± 0.13	3.867 ± 2.678	0.13 ± 0.08
	Stomach	0.162 ± 0.145	3.25 ± 3.71	5.663 ± 3.084	1.82 ± 0.92	9.520 ± 2.808	2.07 ± 1.28
Systemic	Blood	0.050 ± 0.003	16.4 ± 1.6	0.190 ± 0.013	10.1 ± 0.74	0.577 ± 0.104	5.30 ± 0.98
	Heart	0.029 ± 0.009	0.28 ± 0.10	0.089 ± 0.016	0.10 ± 0.06	0.286 ± 0.089	0.07 ± 0.02
	Liver	0.024 ± 0.006	2.81 ± 0.70	0.107 ± 0.005	2.00 ± 0.15	0.290 ± 0.041	0.90 ± 0.10
	Kidney	0.112 ± 0.031	2.39 ± 0.63	0.379 ± 0.040	0.75 ± 0.08	0.902 ± 0.151	0.55 ± 0.09
	Spleen	0.019 ± 0.002	0.11 ± 0.01	0.076 ± 0.005	0.09 ± 0.01	0.207 ± 0.023	0.04 ± 0.01

Quantitation of ^{14}C in the Bloodstream of TDI-Exposed Rats.

Scintillation analysis of whole blood taken immediately following exposure showed that radioactivity reached the bloodstream. Using the calculated total dose, the percentage of the estimated value which was detected in the bloodstream decreased from 16.4 to 5.3 as exposure concentration increased (Table 5). Over the range of exposure concentrations tested, a direct relationship was found between the ppm·hr and the µgEq tolyl group per ml immediate post-exposure terminal blood. The equation of the resultant line is $y = 0.03 + 0.21x$ with an R value equal to 0.985.

Distribution of ^{14}C in Blood Components of TDI-Exposed Rats Analysis of radioactivity in whole blood clearly shows that for all exposures, some form of the labeled compound entered the bloodstream. Biochemical analyses of blood samples were performed to characterize the labelled constituents in the blood immediately following the 4 hour exposures. Plasma and cell components were separated and subjected to scintillation analysis. Table 6 shows the results expressed as a percentage of total blood radioactivity. At all concentrations of TDI tested, the majority of radioactivity was found to be plasma-associated; however, the amount of radioactivity in the cell pellet fraction was measurable and increased with exposure concentration.

TABLE 6: Distribution of Radioactivity in Blood Components

Exposure Concentration (ppm)	Whole Blood Total µgEq	Plasma µgEq (% of Total)	Cell Pellet µgEq (% of Total)
0.026	0.245	0.215 (79)	0.049 (20)
0.143	1.219	0.957 (79)	0.243 (20)
0.821	4.262	3.144 (74)	0.485 (11)

Distribution of Plasma Radioactivity as a Function of Molecular Weight.

One of the primary questions regarding the fate of isocyanates in the blood following exposure is whether there are low molecular weight compounds (eg. TDI, oligoureas or metabolites) and/or high molecular weight adducts. To address this question, plasma samples were subjected to molecular fractionation using Centricon-10 microconcentrators which separate the high molecular weight (>10kDa) conjugates from low molecular weight (<10kDa) conjugates and metabolites. The distribution of radioactivity in the retentate (>10 kDa) and filtrate (<10 kDa) fractions was determined by scintillation analysis (Table 7). The results show that the predominant form (97-100%) of the radioactivity in the plasma immediately following a 4 hour exposure is conjugated material greater than 10 kDa in molecular weight.

TABLE 7: Molecular Sieve Fractionation of Plasma

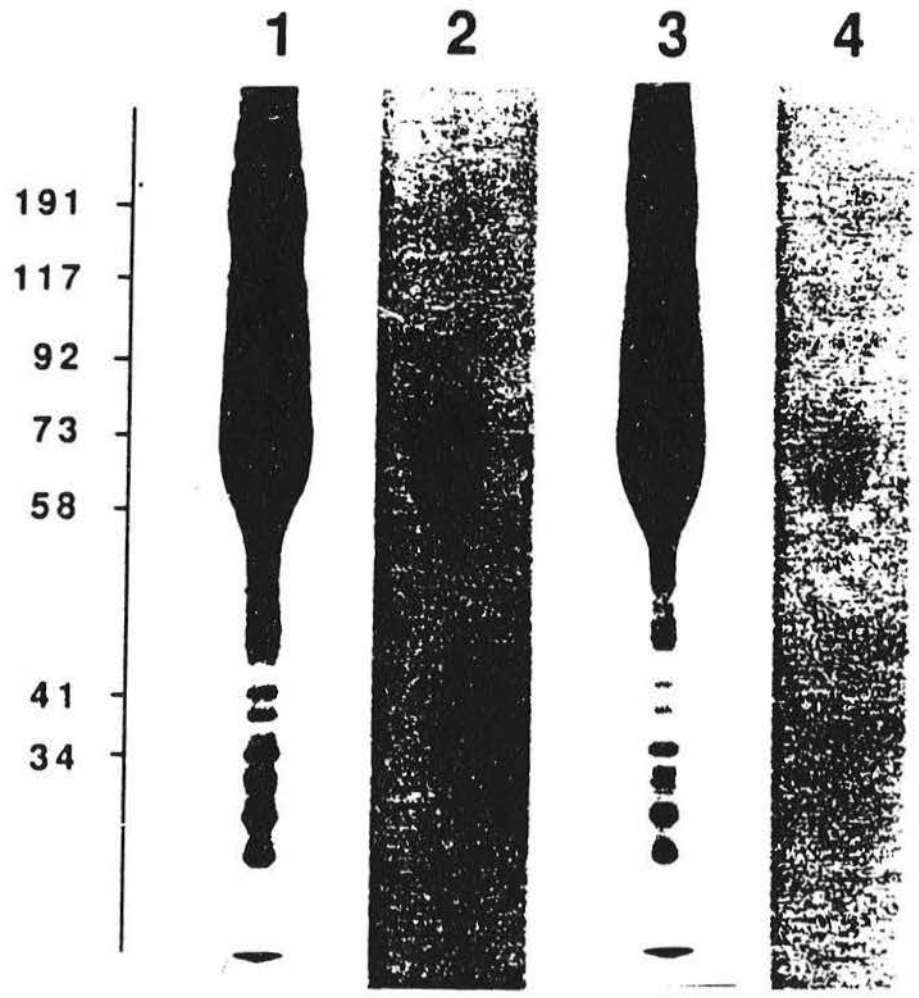
Exposure #	Analysis of Fraction > 10kDa (Retentate Fraction)		Analysis of Fraction < 10kDa (Filtrate Fraction)	
	Total ngEq average ± SD	% of original average ± SD	Total ngEq average ± SD	% of original average ± SD
1	18.4 ± 3.7	100.9 ± 14	0.91 ±0.59	5.1 ± 1.3
2	58.8 ± 7.3	97.0 ± 13	2.41 ± 0.84	3.9 ± 3.9
3	138.0 ± 31.3	100.3 ± 20	5.29 ± 1.41	3.8 ± 1.1

Electrophoretic Analysis of *In Vivo* Conjugates in Plasma.

Plasma retentate (>10kDa) samples were subjected to SDS polyacrylamide gel electrophoresis to further characterize the nature of the *in vivo*, high molecular weight

conjugates. Figure 6 shows the comparative protein profiles for plasma retentates from the two highest TDI exposure groups (Lanes 1 and 3). The distribution of radioactive components in the gel was assayed by autoradiography (Lanes 2 and 4). The majority of radioactivity was associated with a 70 kDa protein band at all concentrations.

FIGURE 6: **(Following Page)** Characterization of Plasma Retentate (>10 kDa) Fractions by SDS Polyacrylamide Gel Electrophoresis and Autoradiography. Coomassie Blue stained gel lanes of plasma retentate samples (>10 kDA) from rats exposed to (0.143 ppm - lane 1; 0.821 ppm - lane 3) are shown. Autoradiographs of the corresponding lanes are shown in lanes 2 and 4, respectively. Molecular weight scale is indicated on left axis based on migration of Sigma SDS-7B standard proteins.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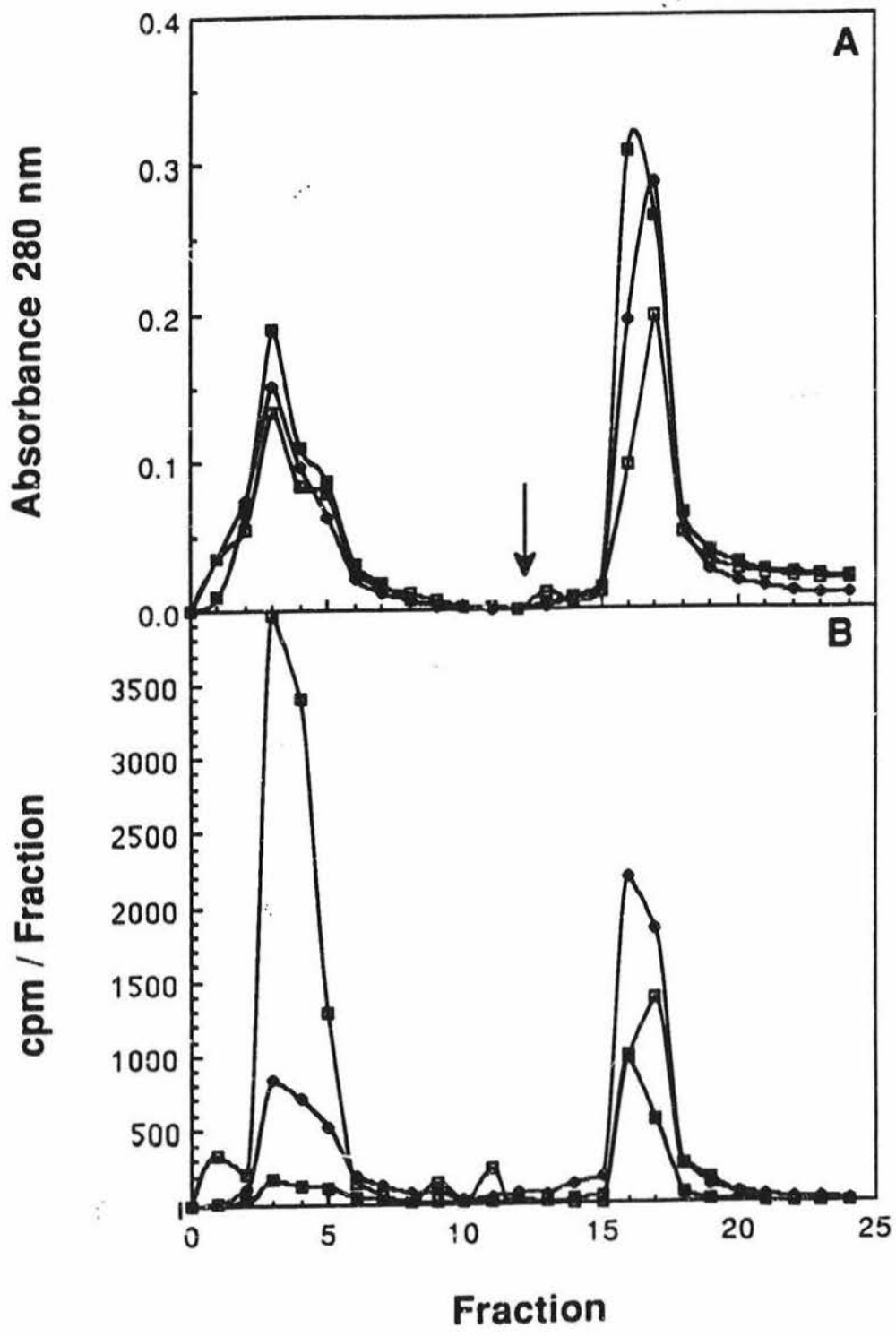
Affinity Chromatography of Plasma Retentate Fractions.

To test if the 70 kDa labeled protein was serum albumin, retentate fractions were run through a Reactive Blue-4 Agarose albumin affinity column. Figure 7A demonstrates that the absorbance profile is quite similar in samples from all three concentration levels tested. In contrast, the corresponding distributions of radioactive components varies with concentration (Figure 7B). Relative to the amount of bound radioactivity, the level of ^{14}C in the unbound peak increases with concentration. This is also supported by the representation of the data given in Table 8 which shows that as concentration increases, the percentage of radioactivity recovered in the unbound fraction also increases.

FIGURE 7: **(Following Page)** Blue Agarose Affinity Chromatography Profile of Plasma Retentates from Rats Following ^{14}C-TDI Exposure. Plasma retentate samples from each of the three exposures were run on a blue agarose, albumin affinity column. Samples were loaded onto the column in 0.01M Tris-HCl, pH 7.8. Peak 1 contains all proteins which did not bind to the column. Bound protein was eluted with a step to a 0.05M Tris-HCl buffer, pH 7.5 containing 0.2M NaSCN. A) Absorbance at 280 nm was monitored for all three exposure concentration samples ((0.026 ppm - open circles; 0.143 ppm - filled triangles; 0.821 ppm - filled squares). B) The total radioactivity in each fraction was also determined.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1000
500
0
5
10
15
20
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TABLE 8. Summary of Blue Agarose Chromatography of Plasma Retentate Fractions

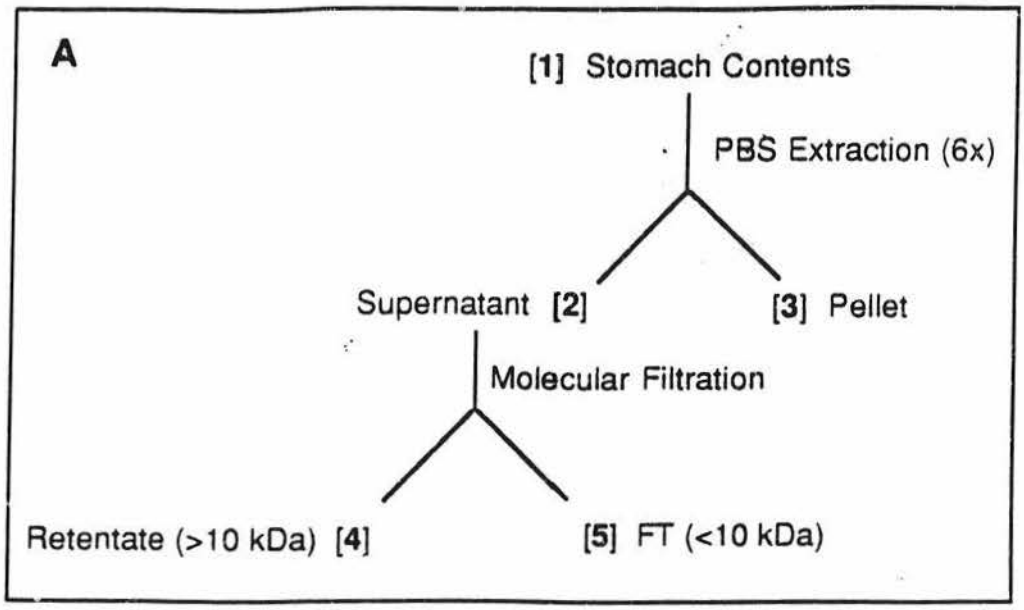
Exposure Conc. (ppm)	Total dpm	Total ngEq	% of total dpm recovered	% of recovered dpm		% of applied dpm	
				Unbound	Bound	Unbound	Bound
0.026	4056	26.8	68	20	68	14	47
0.143	10599 8819	69.9 58.2	86 91	21 31	60 62	18 28	52 56
0.821	19595 18375	129.3 121.2	121 108	48 58	18 20	57 58	20 18

Thin Layer Chromatography of Plasma Filtrate Fractions.

To examine the radioactive material in the low molecular weight filtrate fractions of plasma, thin layer chromatography was performed. In all samples, including control animal plasma filtrate, a species which co-migrated with TDA was detected. Notably, a unique TDI exposure related component was detected by this method. For all lanes, the plate was scraped and silica was subjected to scintillation analysis. The radioactivity was distributed throughout the lane and there was not a single band with a ^{14}C level greater than twice background.

Extraction and Fractionation of Stomach Contents.

In addition to the blood and airway tissues, the other major system which showed an increased level of label was the gastrointestinal tract. To examine the nature of the radioactive material in this system, an aqueous extraction of stomach contents from controls and the highest exposure concentration group was performed and analyzed as illustrated in Figure 8A. The count distribution for each fraction, given in Figure 8B, shows that the majority of the ^{14}C was extractable in the saline wash and that 41% of the material was recovered as high molecular weight conjugates (>10kDa) and 28% was recovered in the filtrate (<10kDa) fraction. The percentage of material in the low molecular weight fraction is increased compared to the similar fraction in plasma.



B

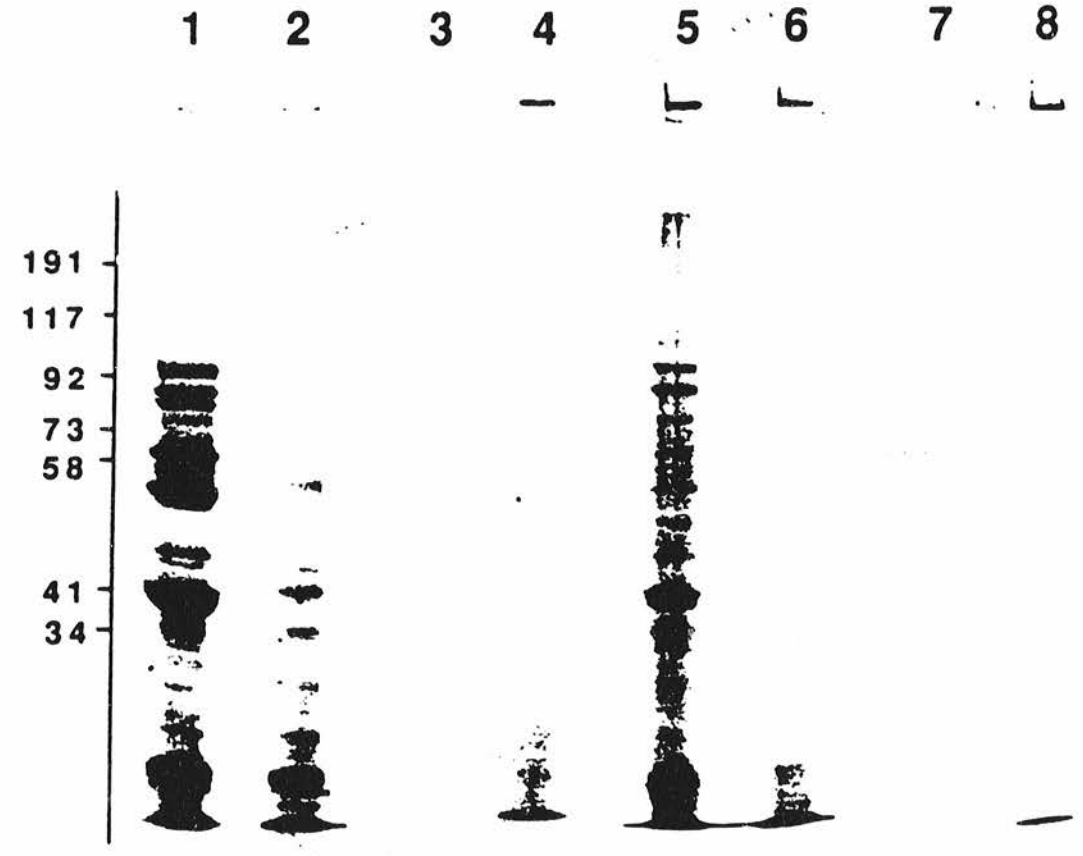
Sample #	Name	Total µgEq	% of Sample # [1]
[1]	Contents	1.212	100
[2]	Supernatant	0.931	77
[3]	Pellet	ND	ND
[4]	Centricon Retentate	0.494	41
[5]	Centricon FT	0.338	28

FIGURE 8: Extraction and Fractionation of Stomach Content Samples Following ^{14}C-TDI Exposure. A) Stomach contents from rats from the control and the high exposure group (0.821 ppm) were extracted and fractionated as diagrammed (Fractions 1-5). B) Total radioactivity for each fraction was determined by scintillation analsyis. The values were converted to µgEq.

Electrophoretic Analysis of *In Vivo* Conjugates in Stomach Content Retentate Fractions.

Retentate (>10kDa) fractions from stomach content extracts were subjected to SDS polyacrylamide gel electrophoresis to further characterize the nature of the *in vivo*, high molecular weight conjugates. Figure 9 shows the comparative protein profiles for the retentates (Lanes 1 and 2) and pellets (Lanes 5 and 6) from the control and 0.821 ppm exposure groups, respectively. The distribution of radioactive components in the gel was assayed by autoradiography (Lanes 3, 4, 7 and 8). Two predominant bands of radioactivity were observed in the retentate fraction, one at the well (> 200 kDa) and one co-migrating with the dyefront (<15 kDa) (Lane 4). In addition, a smear of other labeled products is observed throughout the lane. These high and low molecular weight bands were additionally observed in the pellet fraction as well (Lane 8).

FIGURE 9: **(Following Page)** Characterization of Stomach Content Extract Retentate Fractions by SDS Polyacrylamide Gel Electrophoresis and Autoradiography. Coomassie Blue stained gel lanes of of stomach content extract samples from the control and high exposure group (0.821 ppm) rats are shown. Analyzed fractions include retentates (0 ppm - lane 1; 0.821 ppm - lane 2) and washed pellets (0 ppm - lane 5; 0.821 ppm - lane 6),. Autoradiographs of the corresponding lanes are shown in lanes 3, 4, 7 and 8, for each fraction respectively. Molecular weight scale is indicated on left axis based on migration of Sigma SDS-7B standard proteins.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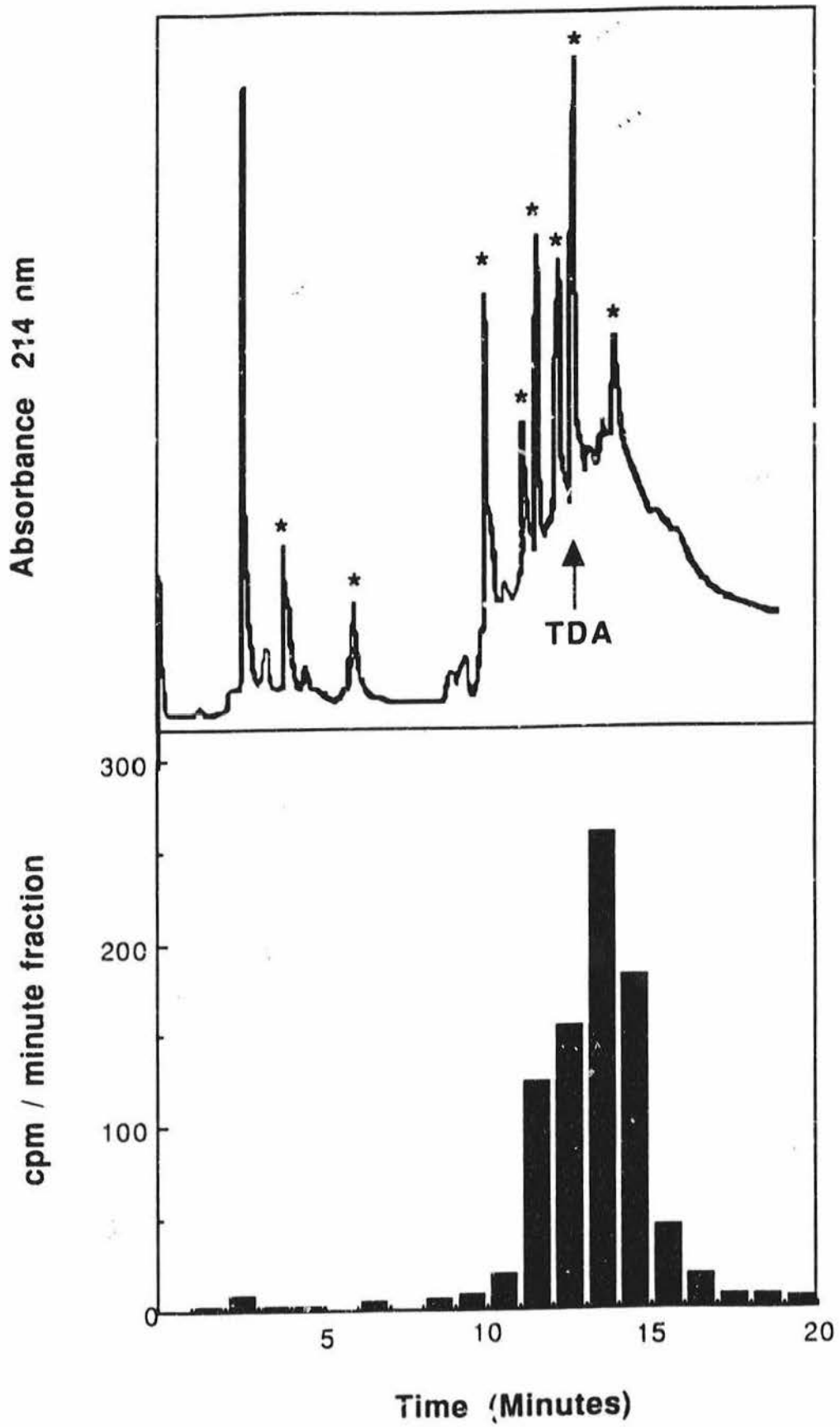
High Pressure Liquid Chromatography (HPLC) of Stomach Content Filtrate Fractions.

Reverse phase HPLC analysis of the stomach content filtrate fractions (Sample [5], Figure 8) was done to examine the distribution of low molecular weight radioactive components. A primary question addressed was whether the low molecular weight form of ^{14}C was TDA, a conversion which may be favored under the acidic conditions of the GI tract. As shown in Figure 10, radioactivity was found not only associated with a TDA co-migrating species but also in numerous other products spread throughout the profile as indicated by the asterisks.

FIGURE 10: (Following Page) Reverse Phase HPLC Analysis of Stomach Content Extract Filtrate Samples Following ^{14}C-TDI Exposure. HPLC analysis of stomach content extract filtrate fractions was performed on a Econosil C_{18} column. A linear gradient was run from 0-80% methanol. Both loading and elution buffers contained 2% PIC B_7 additive. A) Absorbance was monitored at 214 nm. B) Fractions were collected across the gradient at 30 sec intervals and were counted to determine the distribution of radioactivity. Individual peaks were also collected and counted. Radioactivity was associated with numerous absorbing peaks as indicated by asterisks. Migration of injected TDA standard is shown by arrow.


Absorbance 214 nm
TDA
cpm / minute fraction
300
200
100
0
5
10
15
20
Time (Minutes)

DISCUSSION

An inhaled compound may be retained and metabolized in the airways, it may pass directly or by a facilitated mechanism be delivered to the bloodstream, or thirdly, it may be cleared through the mucocilliary transport system. One or more of these processes may contribute to the metabolism and eventual fate of the compound (Dahl, 1988). For a biologically reactive compound, the complexity increases. Therefore, an essential component in understanding a reactive compound's toxicity and estimating associated risks is to characterize its uptake, distribution and *in vivo* reactions. The objective of the present study is to begin to characterize the biochemical events involved following inhalation exposure of rats to ^{14}C TDI vapors.

TDI is a highly reactive compound which possesses the ability to react with several functional groups found on a variety of biological macromolecules. These groups include: amino, hydroxyl, carboxyl, imidazole and sulfhydryl groups (Brown, *et al.*, 1987). Based on the availability of these nucleophilic sites throughout the airway, it is conceivable that TDI would be completely scrubbed directly upon entry. As evidenced in this study (Figure 5, Table 5) this is not an exclusive reaction. The highest levels (μgEq/ml) were, in fact, found in the airway tissues. however, over the concentrations tested, some form of the radioactive compound also penetrated throughout the system.

Analysis of blood samples taken immediately upon termination of the 4 hr exposure showed that by some mechanism, the ^{14}C entered the bloodstream and that the level increased linearly as a function of exposure concentration. This uptake is similar to that observed in the guinea pig model (Kennedy, *et al.*, 1989). A review of blood values in the literature for several species following TDI inhalation exposure is compiled in Figure 11. The linear relationship between tolyl group uptake and exposure concentration is extended over a broad range of TDI concentrations and species even though the method of tolyl group measurement as well as, the TDI isomeric forms used are variable between studies. An interesting point is the plasma value obtained from

human samples (Skarping *et al.*, 1991) which falls on the curve of compiled data from animal studies.

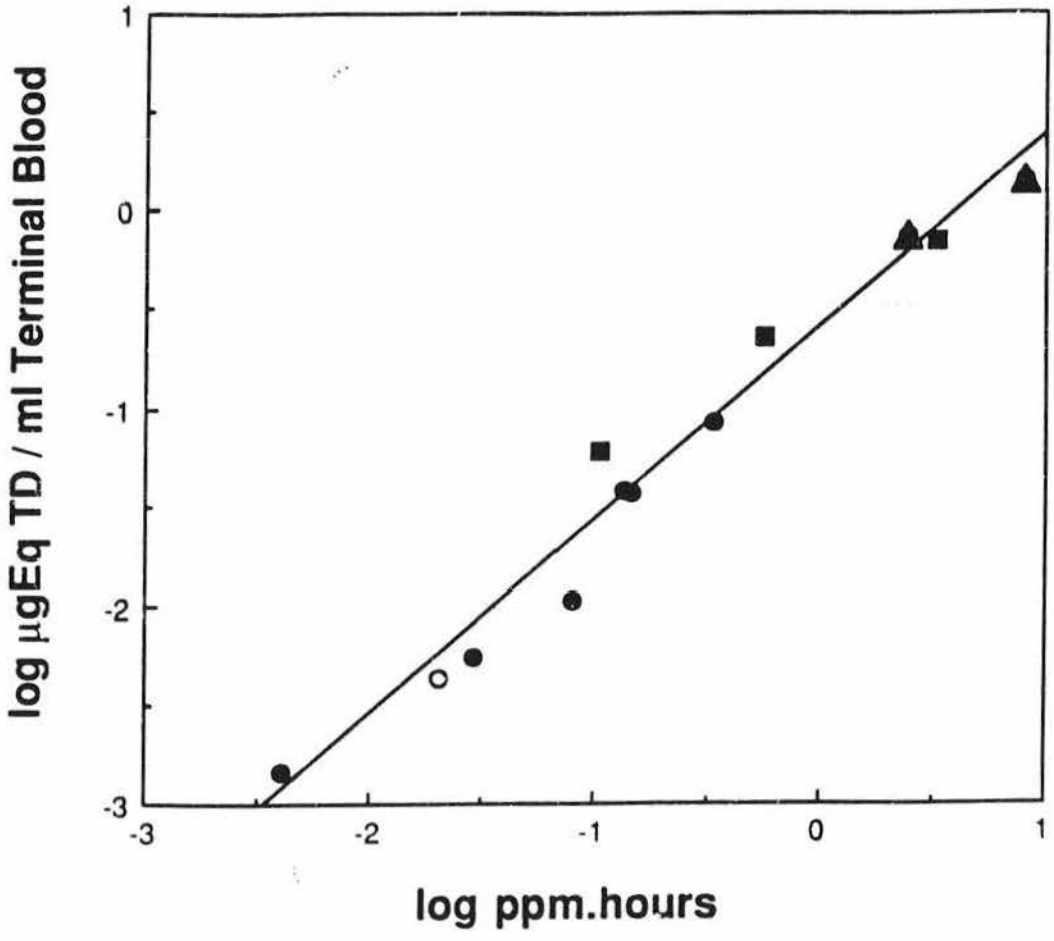


FIGURE 11: Species Comparison of the Level of TDI-Derived Material in the Blood Following Inhalation Exposure. Compilation of values expressed as microgram equivalents of tolyl group in the blood from guinea pigs (closed circles; Kennedy *et al.*, 1989), from rats (closed triangles; Stoltz *et al.*, 1987, closed squares; this study) and in a human plasma sample (open circle; Skarping *et al.*, 1991)

In addition to understanding that the radioactivity, in some form, is taken into the bloodstream, it is perhaps more important to investigate the biochemical state of this radioactivity. One of the potential routes of uptake of TDI or its products into the bloodstream could be direct penetration from the respiratory tract surfaces into the blood. Based on the high degree of reactivity described above, this possibility appears unlikely. Another potential mechanism would involve the reaction of TDI within the aqueous environment of the airway which could result in hydrolysis and diamine formation. Thirdly, the isocyanate could react, in the airway, to form adducts with peptides, proteins, lipids and carbohydrates which then could be transferred into the bloodstream. To investigate these possibilities, biochemical analyses of the ^{14}C-material in terminal blood was performed. The first level of characterization was to determine the distribution of ^{14}C in the various blood components. For all exposure concentrations tested, the majority of radioactivity was plasma-associated (Table 6). Analysis of the cell-associated material evidenced hemoglobin adduct formation (G. Sabionni; personal communication). Further analysis of the plasma by molecular weight fractionation was also performed. The rationale for this separation was that if free TDI or TDA were the form of the labeled material in the bloodstream, the ^{14}C would be associated with the tolyl group and would be of a low molecular weight. Alternatively, if biomolecular adducts were formed, the molecular weight of the ^{14}C adduct would be significantly higher. The results showed that greater than 95% of the plasma-associated radioactivity existed in a conjugated form with a molecular weight greater than 10 kDa (Table 7). This data suggests that the *in vivo* reaction of TDI with biological macromolecules successfully competes with hydrolysis to the diamine. However, in the timeframe of the exposures, it is possible that hydrolysis to the diamine occurred. The diamine could have then been activated, either locally or systemically, to form a compound capable of *in vivo* reaction. Such an activation could also account for the high molecular weight components observed. Methods are currently being developed and tested to distinguish

between the products of direct or indirect conjugation reactions. Regardless of the pathway, the results support the conclusion that conjugation predominates under the experimental conditions tested.

Electrophoretic characterization of the retentate fractions from plasma demonstrated a degree of specificity in the conjugation reactions. At all concentrations of TDI tested, the predominant radioactive component detected was a protein of 70 kDa molecular weight (Figure 6). Due to the relative abundance of serum albumin in plasma and the similarity in molecular weight, it was hypothesized that the ^{14}C-labeled 70 kDa protein was TDI-modified serum albumin. An albumin affinity column was run and the profiles supported the idea that some portion of the labeled material was the modified albumin (Figure 7). However, at the highest concentration, it appears a threshold of labeling is reached where upon another component is labeled which does not bind to the column. Alternatively, it is also possible that so many molecules of TDI had reacted with the albumin molecule that its binding affinity was altered (Figure 7, Table 8). The *in vivo* modification of an albumin-like protein by TDI has been shown for human samples as well (Kochman, *et al.*, 1990; Baur, *et al.*, 1992). The role of such a conjugate in either species has not yet been determined. It appears from this study and the work of others, that the 70 kDa protein, as well as other biomolecular conjugates of TDI are the predominant form of the compound following inhalation exposure. Skarping and co-workers have hydrolyzed human plasma and urine samples from individuals which have been exposed to TDI. Under the harsh conditions of acid hydrolysis used in the analysis of biological fluids from exposed humans, TDI conjugate bonds would be broken and could yield TDA as a reaction product. They have reported that TDA can be quantitated at a level related to exposure dose but that free TDA could not be detected prior to hydrolysis (Skarping *et al.*, 1991). These observations are particularly important with regard to the recent interest in the potential carcinogenicity of TDI. Studies investigating the carcinogenicity of TDI have shown that it is not carcinogenic following inhalation

(Loeser, 1983); however, tumor production following gavage administration has been observed and is dose dependent (Dieter *et al.*, 1990). The tumors observed included: subcutaneous fibrom , fibrosarcomas, pancreatic islet cell adenomas, neoplastic nodules of the liver and mammary gland tumors. Studies with toluenediamine (TDA), the hydrolysis product of TDI, have confirmed it to be carcinogenic in rodents (National Cancer Institute, 1979). Therefore, the metabolic fate of TDI *in vivo*, particularly its hydrolytic conversion to TDA, is important in regard to assessment of risk. Studies on the metabolism of TDI following gavage administration have shown that TDA and subsequent metabolic products are present in the urine of exposed animals (Dieter *et al.*, 1990; Timchalk, *et al.*, 1993). It has been hypothesized that upon delivery to an acidic environment such as the conditions of the gastrointestinal tract, the hydrolysis of TDI to TDA would be favored over conjugate formation (Brown and Kennedy, 1990). In this study, a significant fraction of the radiolabeled material was found associated with the tissues and materials of the gastrointestinal tract (Figure 5; Table 5). Extraction and biochemical analysis of the stomach contents was performed to investigate the state of the ^{14}C material. Molecular weight fractionation studies showed that just as seen with the plasma, a conjugated form of the radioactive material predominated, however, a larger amount of low molecular weight material was observed (Figure 8). Based on the lower pH of the stomach, if reactive TDI reached this compartment, hydrolysis to the diamine would be predicted. This could occur by gasping and swallowing air. Alternatively, the material in the stomach could have first entered the airway and then could have been delivered through mucocilliary clearance, to the stomach. HPLC characterization of the filtrate fraction of the stomach content extract demonstrated that the low molecular weight fraction had numerous radiolabeled components (Figure 10). A TDA co-migrating species was observed but was only one among a variety of other products. The prevalence of adducts suggests that the primary reactions occurred in the airway where the protonation state of the macromolecules would favored nucleophilic

reactions. The low molecular weight of these products may be attributable to proteolysis once the conjugates entered the stomach environment. These results do not parallel the polymerization and hydrolysis that was seen by gavage administration of TDI (Jeffcoat,1985; Timchalk, *et al.*, 1993). This asserts that especially for reactive chemicals, the route of administration may severely influence the compound reactivity and subsequent fate.

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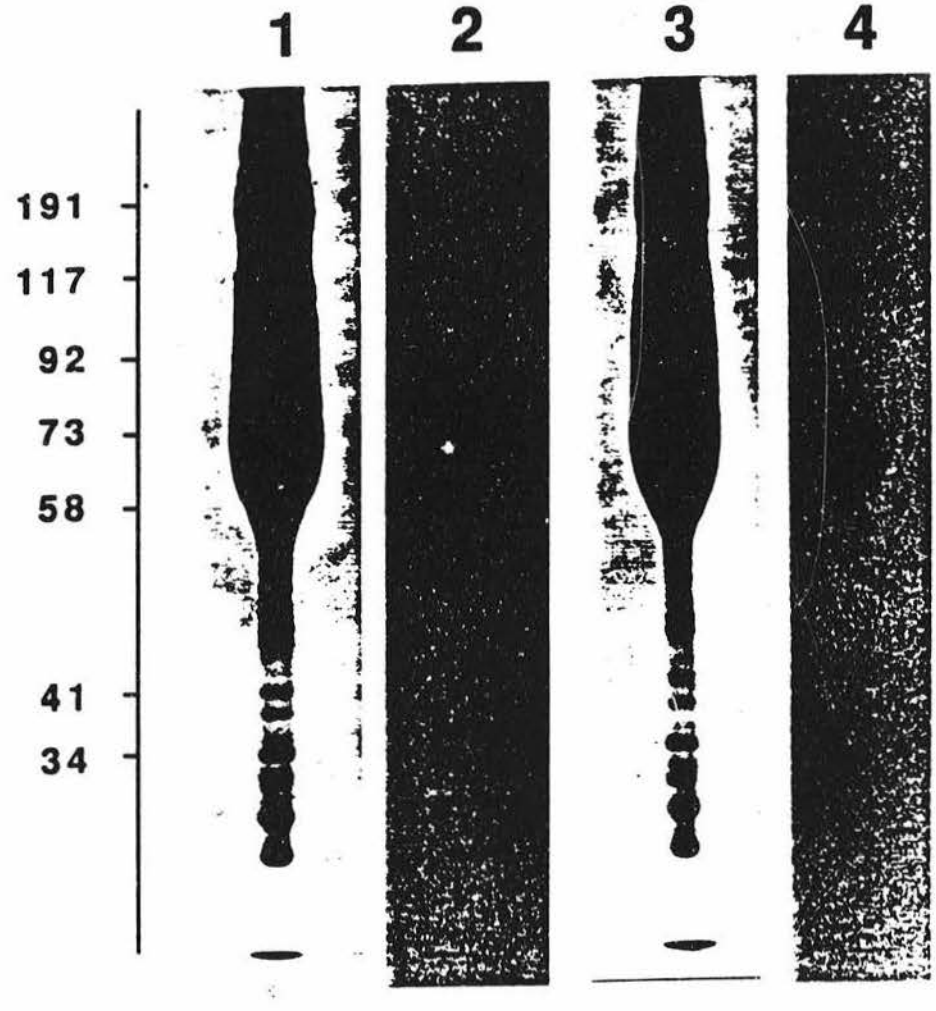
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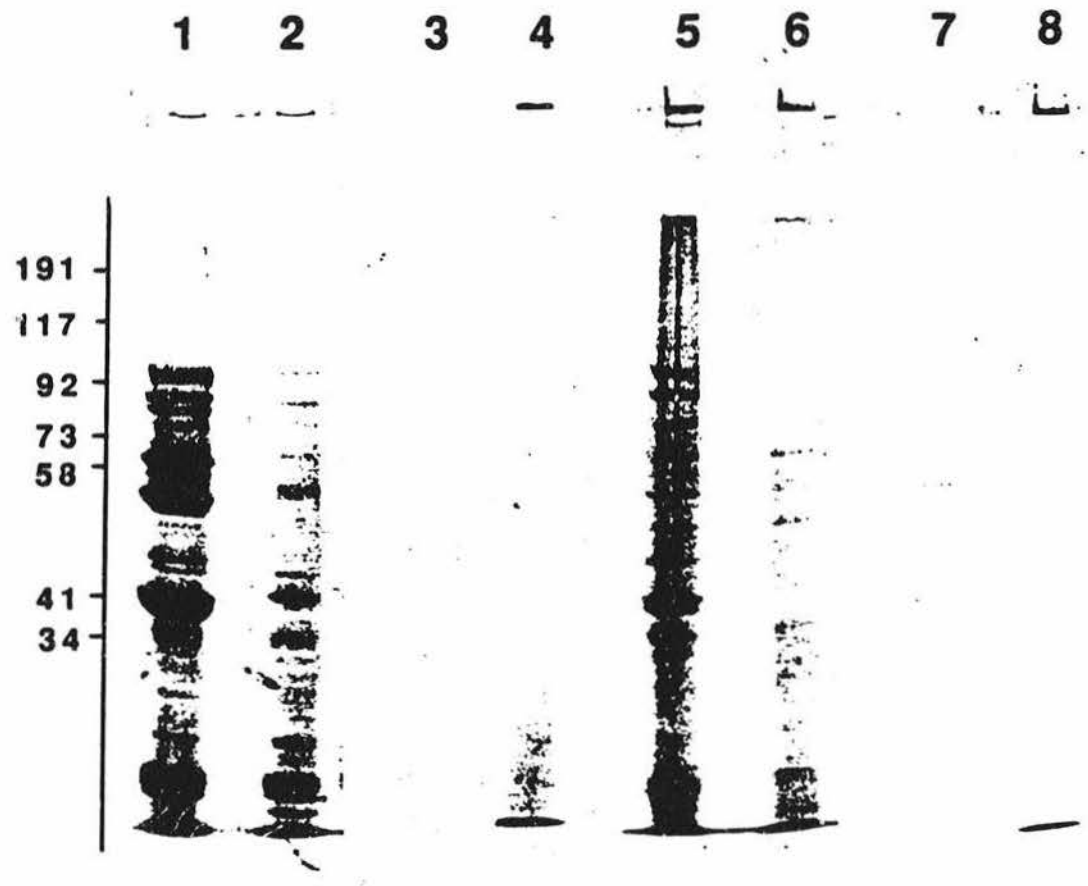
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